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# TNF-family member Receptor Activator of NF- $\kappa$ B (RANK) and RANK-Ligand (RANKL) in bone remodelling

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**Abstract.** Receptor Activator of NF- $\kappa$ B (RANK) and RANK-Ligand (RANKL) are members of the Tumour Necrosis Factor (TNF)-superfamily involved in bone homeostasis. In a tightly controlled interplay of this receptor and ligand, bone is continuously being remodelled. Several pathological conditions resulting from a misbalance between bone resorption and bone formation have been documented. Most frequently resorption gets the overhand resulting in a lower Bone Mineral Density (BMD), increased fracture risk and reduced mobility of patients. RANKL is expressed on bone producing osteoblasts whereas RANK is expressed on pre-osteoclasts, which can develop in bone resorbing osteoclasts. The binding of RANKL to RANK functions as a trigger for the formation of these bone resorbing osteoclasts. With the development of a monoclonal antibody directed against RANKL a new therapeutic strategy to interfere with bone remodelling has become available some years ago. In this manuscript we discuss the prospective of interfering with the RANK/RANKL pathway as a therapeutic target for bone diseases. We discuss the role of the soluble receptor osteoprotegerin (OPG) as a therapeutic in bone diseases. Then we focus on the possibility to develop antagonistic and agonistic variants of RANKL based on computational protein design and we discuss the development of antagonistic RANKL variants by changing the stoichiometry of the RANKL molecule.

## 1. Introduction

The Tumor Necrosis Factor (TNF) - superfamily is a group of cytokines consisting of ligands and receptors whose interactions regulate cell differentiation and controlled cell death (apoptosis) [1]. Thanks to their essential role in cell survival, many TNF-superfamily members are being investigated as potential therapeutic targets for diseases as diverse as cancer [2] and rheumatoid arthritis [3]. TNF-superfamily members Receptor Activator of NF- $\kappa$ B (RANK), RANK Ligand (RANKL) and osteoprotegerin (OPG) have a role in proliferation of bone cells as they control the balance between bone producing osteoblasts and bone resorbing osteoclasts, thereby forming the human skeleton [4].

From its appearance the skeleton seems to be a static structure, but bone tissue is continuously being remodelled. The complete human skeleton is renewed in approximately 10 years [5]. Remodelling is necessary to adapt to changes due to mechanical loads [6]. These lead to micro damage, which needs to be repaired to maintain the strength of the skeleton [7]. Differences in inter individual pressure on the skeleton lead to differences in bone remodelling. Individuals who exercise more and have more physical activity have a lower risk of fractures. Age related thinning of the bone



is associated with an increase in fragility of bone, which may result in fractures upon falling. By aging of our population bone diseases become more frequent [8].

Bone diseases can be classified as syndromes with a lowered bone mass, with an increased bone mass and as bone tumours. In osteoporosis, there is an imbalance between osteoblastic and osteoclastic activity in favor of osteoclastic activity, leading to bone fragility and an increased risk of fractures [9]. Paget's disease is the most common bone disease in which there is an increased bone mass. It is characterized by deformation of the bone. Usually there are one or more focal areas of disorganized bone remodelling. Following osteoporosis, it is the most frequent bone disease [10]. Multiple myeloma is the most common primary malignancy of bone with marrow infiltration of the skeleton, which produces widespread osteolytic bone damage. In multiple myeloma, the balance between osteoblastic and osteoclastic activity is disturbed in favor of osteoclastic activity [11].

Recently, the first biological -denosumab, an antibody directed against RANKL- was introduced and accepted by legal authorities for treating osteoporosis. The monoclonal antibody (mAB) interferes with the RANK/RANKL signalling and thereby inhibits not only osteoclast activity [12] but also bone tumours [13]. Recent discoveries such as the structure of RANKL bound to RANK and OPG have given further insight into the molecular mechanisms of RANK/RANKL and OPG in bone remodelling, elucidating key residues interaction of the ligand and the receptors [14-17].

This review will focus on strategies to combat bone diseases making use of the RANK/RANKL pathway. We will discuss the process of RANK/RANKL controlled bone remodelling in the next paragraph and will thereafter discuss mechanisms of action of (new) therapeutic options to treat bone diseases interfering with this pathway.

## **2. RANK/RANKL/OPG controlled bone remodelling**

Osteoblasts originate from pluripotent mesenchymal cells. Osteoblast cells are responsible for the formation, deposition and mineralization of bone tissue [18]. Bone is an important source for calcium and phosphate and as such important for mineral homeostasis [19]. Parathyroid Hormone (PTH) is a key regulator of calcium metabolism. When blood calcium levels are reduced, PTH is produced and secreted thereby increasing osteoclast mediated bone resorption which increases calcium levels [20].

RANK and its corresponding ligand RANKL play a role in bone homeostasis by tightly controlling the balance between bone resorbing osteoclasts and bone producing osteoblasts. Upon activation by PTH, RANKL is presented on the surface of osteoblasts [5]. RANK is presented on the surface of pre-osteoclasts upon binding of a paracrine factor, Macrophage Colony Stimulating Factor (M-CSF) [21]. Osteoblasts secrete M-CSF, which in turn binds to its corresponding receptor M-CSFR on pre-osteoclasts activating the RANK/RANKL pathway [22].

Interaction between RANKL and RANK leads to fusion of the osteoclast precursors resulting in multinucleated mature osteoclasts having the capability to resorb bone. Upon RANKL binding to RANK, TNF receptor associated factor-1 (TRAF1), -2, -3, -5, and -6 are recruited to the intracellular domain of RANK [23]. TRAF6 seems to be the most important of these as recruitment leads to the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) [24]. NF- $\kappa$ B in turn regulates transcriptional activity of osteoclast-specific proteins [25]. Osteoprotegerin (OPG) acts as a decoy receptor for RANKL thus preventing interaction between RANK and RANKL resulting in less fusion of the OPCs [26]. The RANK-RANKL pathway is important in bone development in both physiological and pathological situations. Therefore, this signal pathway can be considered as a promising target for bone-related diseases [27].

## **3. Interfering with the RANK/RANKL pathway in combatting bone diseases**

### **3.1. Antagonizing RANKL**

The natural antagonist for suppressing RANKL titers is OPG. This soluble receptor of the TNF family is present at high concentrations in bone and it plays a natural role in protecting bone mass, hence, its name osteoprotegerin. OPG and RANK bind to slightly different regions of RANKL. RANK distributes its contacts evenly around the DE-loop and A'A''-loop of RANKL. In contrast the contacts made by OPG are limited to the E-strand and DE-loop region of RANKL, almost completely avoiding the A'A''-loop region [14]. OPG exerts its function as decoy receptor by blocking residues essential for RANKL to bind RANK [28]. Initial trials to use OPG as a therapeutic have failed due to the poor pharmacokinetic and pharmacodynamic properties and due to interference with the heparin binding domain in mice [29]. As a result, numerous fusion proteins containing a variety of residues of human OPG fused to human Fc fragments were tested and some were found to be 200 times more active than full-length OPG in vivo. A recombinant Fc-OPG, AMGN-0007, was in phase I clinical study for osteolytic bone metastasis and was discontinued due to the potential safety risk of immune response [30]. The human RANKL binding domain of RANK fused Fc fragment (RANK-Fc) was also found to be more effective than native OPG. However, in primates, following repeated dosing of human RANK-Fc, inactivating autoantibody titers against RANK were detected and the development was discontinued. Subsequently, monoclonal antibodies were selected and one monoclonal antibody obtained from immunization of the IgG2 XenoMouse emerged as a potent inhibitor of RANKL induced osteoclast formation. The resulting monoclonal antibody (an IgG2 $\kappa$  antibody) was originally designated AMG 162 and is now known as denosumab. Alternatively, peptide mimics of OPG (OP3-4 peptide) [31], RANK [32] and the tumour necrosis factor (TNF) receptor (WP9QY peptide) [33] were also developed. Although they showed inhibitory activity against the RANKL-induced osteoclastogenesis, further research of them is needed before these can be applied for therapeutic treatment.

The use of OPG in bone malignancies is limited as OPG also functions as a decoy receptor for Tumour Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) as well, preventing the apoptosis of tumour cells [34]. Therefore, structure-based OPG mutants have been designed that still do antagonize RANKL inhibiting osteoclastogenesis, but which do not bind TRAIL and therefore do not interfere with tumour apoptosis [34]. TRAIL cancer therapy has been shown to be effective. Recombinant human TRAIL variants with a decreased affinity for OPG have been investigated and this research was done to overcome TRAIL resistance when applied in the bone environment [35].

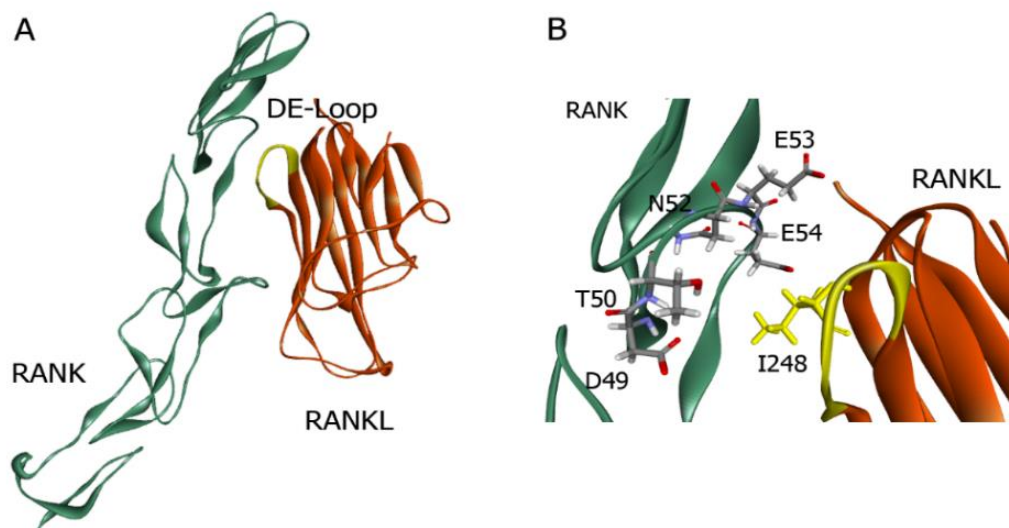
### 3.2. Antagonizing RANK

One other approach to target bone diseases is to target and antagonize RANK directly. The advantage of targeting the receptor over targeting the ligand RANKL can be found in the direct inactivation of the receptor. Although denosumab titrates away RANKL, it does not block the receptor. Furthermore the administration of antibodies such as denosumab carries the risk of immune reactions [36]. Compared with antibody, administration of structure-based protein mutants are most likely to trigger the auto immune system with less impact, as the mutations in the proteins involve less residues. Therefore, the design of antagonistic proteins based on the RANKL seems to be an attractive approach.

The feasibility to design variants of TNF-ligands with altered binding properties was first shown for TRAIL [37]. In many cancer cells, apoptosis seems to occur primarily via only one of the two TRAIL death receptors and the introduction of receptor selectivity is a way to create more potent TRAIL agonists. With computational protein design it is possible to predict protein segments and amino acids important for binding, affinity and strength of the ligand-receptor complexes [38]. All residues in the receptor binding interface can be mutated into any possible other amino acid. A set of predicted energetic values for the wild type TRAIL - receptor complex formation was compared with the obtained energetic values after mutagenesis. The change in binding energy at every residue can be used as a prediction for improved or decreased binding. Afterwards predicted variants were expressed,

purified and screened for receptor selectivity by surface plasmon resonance (SPR). TRAIL variants selective for a specific TRAIL death receptor could be obtained this way [37, 39-40].

Due to the high homology among TNF ligands and their cognate receptors, an alternative strategy to predict variants with agonistic or antagonistic properties is to compare different TNF-superfamily members; interaction domains and interacting residues do show similarity among TNF ligand-receptor complexes. One of these interaction loops is the so called DE-loop. There is a conserved tyrosine in the DE-loop of all TNF-ligands except CD40L and RANKL. Shibata and others found that mutations in this hydrophobic residue of the DE-loop in TNF- $\alpha$  yielded TNF-receptor 1 selective antagonist [41]. The 3D structure of the complex between RANK and RANKL suggests that hydrophilic interactions are predominant among all the surface interactions between the DE-loop of RANKL and RANK. However, RANKL mutation at the equivalent residue 248 from isoleucine to aspartate resulted in an eightfold lower activity [16]. Then, the effect of further mutations at position I248 in the DE-loop of murine RANKL on the interaction of RANKL with RANK was investigated. RANKL I248Y and I248K were found to compete with wild-type RANKL binding to RANK while reducing wild-type RANKL-induced osteoclastogenesis [42]. These mutations suggest that the DE-loop of RANKL is important for osteoclastogenesis and two newly formed mutants, RANKL I248Y and I248K, can offer a starting point for novel therapeutic proteins in new osteoporosis treatments (Figure 1)



**Figure 1.** Structural overview of the area surrounding the DE loop of (A) Refined structure of RANKL (orange) -RANK (green) complex. The DE loop of RANKL is shown in yellow. The figure was derived from the known structure of RANK: RANKL complex (PDB ID: 4GIQ). (B) Close-up view of residues near I248 in the DE-loop of RANKL. Changes in the stoichiometry of the receptor.

### 3.3. Changes in the stoichiometry of the receptor

An important characteristic of the TNF superfamily is the multimeric organization of receptors and ligands. For RANK and RANKL a trimeric RANK receptor binds to a trimeric RANKL ligand; this is needed for the biological effect [43]. RANK trimerizes through hydrophobic interactions at the core of the trimer, leading to assembly of a homotrimer [16]. However, TNF ligands can also bind to their corresponding monomeric and dimeric receptor subunits. It was found that there is a high affinity for the first binding to a monomeric subunit of the receptor. Using Surface Plasmon Resonance a high affinity binding between RANKL and the first monomeric subunit of RANK was demonstrated. A



dissociation constant ( $K_D$ ) of 0.67 nM with  $k_a=4.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  and  $k_d=3.3 \times 10^{-4} \text{ M}^{-1}\text{s}^{-1}$  could be calculated [44].

The development of RANKL variants occupying only one monomer of the RANK receptor obstructing the binding to three monomeric RANK receptors will have an antagonistic effect on osteoclast formation [45]. By creating RANKL variant that only has a single intact receptor binding interface, one RANK receptor might still be bound but additional receptor molecules can no longer be recruited to form the biologically active trimeric receptor complex. This leaves less receptor molecules available to trimerize. Thus, these variants do not only block RANK-RANKL interaction by competition with trimeric RANKL but also leave less RANK receptor molecules available on the cell surface.

Interestingly, monoclonal antibodies, such as denosumab, bind to trimeric TNF family members mostly in a 3:2 complex, meaning 3 denosumab antibody molecules bind two RANKL molecules (trimeric), resulting in complicated kinetics [46]. Compared with the antibodies, structure-based variants binding in a 1:1 complex might exhibit simpler kinetics resulting in a reduction of the risk of side effects.

#### 4. Conclusion

The TNF-superfamily members RANKL and RANK are important for bone remodelling. Their roles in osteoclast derived bone resorption have been the subject of study for a long time. With the development of a monoclonal antibody derived against RANKL, a new therapeutic approach to combat bone diseases became available. In the review we presented three ways to develop new possible therapeutics to interfere with RANK/RANKL signalling. First, we focused on the possibility to use OPG, the natural decoy receptor for RANKL. Then we discussed the development of RANKL variants with antagonistic properties, based on computational design using conserved domains in the TNF-superfamily. Finally, we discussed antagonistic molecules interfering with the trimerization of RANK, a conformation essential for signal transduction. Interfering with the RANK/RANKL pathway is a promising strategy to develop therapeutics to combat bone diseases.

#### References

- [1] Aggarwal B B, 2003, Signalling pathways of the TNF superfamily: A double-edged sword, *Nature Reviews Immunology*, **3**: 745–56
- [2] Azijli K, Weyhenmeyer B, Peters G J, De Jong S and Kruyt F A E, 2013, Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: Discord in the death receptor family, *Cell Death Differ.*, **20**: 858–868
- [3] Vinay D S and Kwon B S, 2012, Targeting TNF superfamily members for therapeutic intervention in rheumatoid arthritis, *Cytokine*, **57**: 305–312
- [4] Yasuda H, 2013, RANKL, a necessary chance for clinical application to osteoporosis and cancer-related bone diseases, *World J. Orthop.*, **4**: 207
- [5] Manolagas S C, 2000, Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis., *Endocr. Rev.*, **21**: 115–37
- [6] Cameron J A, Milner D J, Lee J S, Cheng J, Fang N X and Jasiuk I M, 2013, Employing the biology of successful fracture repair to heal critical size bone defects, *Curr. Top. Microbiol. Immunol.*, **367**: 113–32
- [7] Eriksen E F, 2010, Cellular mechanisms of bone remodeling, *Rev. Endocr. Metab. Disord.*, **11**: 219–27
- [8] Brent Richards J, Zheng H F and Spector T D, 2012, Genetics of osteoporosis from genome-wide association studies: Advances and challenges, *Nat. Rev. Genet.*, **13**: 576–88
- [9] Davidson M R, 2003, Pharmacotherapeutics for osteoporosis prevention and treatment, *J. Midwifery Womens. Health*, **48**: 39–52
- [10] Ralston S H, 2013, Paget's Disease of Bone, *N. Engl. J. Med.*, **368**: 644–650
- [11] Tosi P, 2013, Diagnosis and treatment of bone disease in multiple myeloma: spotlight on spinal

- involvement, *Scientifica (Cairo)*, **2013**: 1–12
- [12] Brown J P, Reid I R, Wagman R B, Kendler D, Miller P D, Jensen J E B, Bolognese M A, Daizadeh N, Valter I, Zerbini C A F and Dempster D W, 2014, Effects of Up to 5 years of denosumab treatment on bone histology and histomorphometry: The FREEDOM study extension, *J. Bone Miner. Res.*, **29**: 2051–6
- [13] Shapiro C L, Moriarty J P, Dusetzina S, Himelstein A L, Foster J C, Grubbs S S, Novotny P J, and Borah B J, 2018, Cost-effectiveness analysis of monthly zoledronic acid, zoledronic acid every 3 months, and monthly denosumab in women with breast cancer and skeletal metastases: CALGB 70604 (Alliance), *J. Clin. Oncol.*, **35**: 3949–3956
- [14] Nelson C A, Warren J T, Wang M W, Teitelbaum S L, Fremont D H and Engage R, 2012, RANKL employs distinct binding modes to engage RANK and the osteoprotegerin decoy receptor, *Structure*, **20**: 1971–82.
- [15] Liu C, Walter T S, Huang P, Zhang S, Zhu X, Wu Y, Wedderburn L R, Tang P, Owens R J, Stuart D I, Ren J and Gao B, 2010, Structural and functional insights of RANKL-RANK interaction and signaling, *J. Immunol.*, **184**: 6910–9.
- [16] Lam J, Nelson C A, Ross F P, Teitelbaum S L and Fremont D H, 2001, Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity, *J. Clin. Invest.*, **108**: 971–979.
- [17] Luan X, Lu Q, Jiang Y, Zhang S, Wang Q, Yuan H, Zhao W, Wang J and Wang X, 2016, Crystal structure of human RANKL complexed with its decoy receptor osteoprotegerin, *J. Immunol.*, **189**: 245–52.
- [18] Phan T C, Xu J and Zheng M H, 2004, Interaction between osteoblast and osteoclast: impact in bone disease., *Histol Histopathol*, **19**: 1325–1344.
- [19] Bergwitz C and Jüppner H, 2010, Regulation of phosphate homeostasis by PTH, Vitamin D, and FGF23, *Annu. Rev. Med.*, **61**: 91–104.
- [20] Gaffney-Stomberg E, Lutz L J, Rood J C, Cable S J, Pasiakos S M, Young A J and McClung J P, 2014, Calcium and vitamin D supplementation maintains parathyroid hormone and improves bone density during initial military training: a randomized, double-blind, placebo controlled trial, *Bone*, 46–56.
- [21] Lagasse E and Weissman I L, 1997, Enforced expression of Bcl-2 in monocytes rescues macrophages and partially reverses osteopetrosis in op/op mice., *Cell*, **89**: 1021–1031.
- [22] Boyce B F, Rosenberg E, de Papp A E, and Duong L T, 2012, The osteoclast, bone remodelling and treatment of metabolic bone disease, *Europ. J. Clin. Invest.*, **42**: 1332–41.
- [23] Galibert L, Tometsko M E, Anderson D M, Cosman D and Dougall W C, 1998, The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily, *J Biol Chem*, **273**: 34120–7.
- [24] Darnay B G, Besse A, Poblentz A T, 2007, Lamothe B and Jacoby J J, TRAFs in RANK signaling, *Adv. Experi. Medici. Biol.*, **597**: 152–159.
- [25] Honma M, Ikebuchi Y, Kariya Y and Suzuki H, 2014, Regulatory mechanisms of RANKL presentation to osteoclast precursors, *Curr. Osteoporos. Rep.*, **12**: 115–20.
- [26] Simonet W S, Lacay D L, Dunstan C R, Kelley M, and Chang M S, 1997, Lüthy, R., Osteoprotegerin: a novel secreted protein involved in the regulation of bone density., *Cell*, **89**: 09–319.
- [27] Tanaka S, Nakamura K, Takahashi N and Suda T, 2005, Role of RANKL in physiological and pathological bone resorption and therapeutics targeting the RANKL-RANK signaling system., *Immunol. Rev.*, **208**: 30–49.
- [28] Luan X, Lu Q, Jiang Y, Zhang S, Wang Q, Yuan H, Zhao W, Wang J and Wang X, 2012, Crystal structure of human RANKL complexed with its decoy receptor osteoprotegerin, *J. Immunol.*, **189**: 245–52.
- [29] Bekker P J, Holloway D, Nakanishi A, Arrighi M, Leese P T and Dunstan C R, 2001, The effect



- of a single dose of osteoprotegerin in postmenopausal women., *J. Bone Miner. Res.*, **16**: 348–60.
- [30] Body J J, Greipp P, Coleman R E, Facon T, Geurs F, Feraud J P, Harousseau J L, Lipton A, Mariette X, Williams C D, Nakanishi A, Holloway D, Martin S W, Dunstan C R and Bekker P J, 2003, A phase I study of AMG-007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases., *Cancer*, **97**: 887–92.
- [31] Heath D J, Vanderkerken K, Cheng X, Gallagher O, Prideaux M, Murali R and Croucher P I, 2007, An osteoprotegerin-like peptidomimetic inhibits osteoclastic bone resorption and osteolytic bone disease in myeloma, *Cancer Res.*, **67**: 202–8.
- [32] Ta H M, Nguyen G T T, Jin H M, Choi J, Park H, Kim N, Hwang H Y and Kim K K, 2010, Structure-based development of a receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) inhibitor peptide and molecular basis for osteopetrosis, *Proc. Natl. Acad. Sci.*, **107**: 20281–6.
- [33] Aoki K, Saito H, Itzstein C, Ishiguro M, Shibata T, Blum R, Mian A H, Takahashi M, Suzuki Y, Yoshimatsu M, Yamaguchi A, Deprez P, Mollat P, Murali R, Ohya K, Horne W C and Baron R, 2006, A TNF receptor loop peptide mimic blocks RANK ligand-induced signaling, bone resorption, and bone loss, *J. Clin. Invest.*, **116**: 1525–34.
- [34] Higgs J T, Jarboe J S, Lee J H, Chanda D, Lee C M, Deivanayagam C, and Ponnazhagan S, 2015, Variants of osteoprotegerin lacking TRAIL binding for therapeutic bone remodeling in osteolytic malignancies, *Mol. Cancer Res.*, **33**: 395–401.
- [35] Bosman M C J, Reis C R, Schuringa J J, Vellenga E and Quax W J, 2014, Decreased affinity of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) D269H/E195R to osteoprotegerin (OPG) overcomes trail resistance mediated by the bone microenvironment, *J. Biol. Chem.*, **289**: 1071–8.
- [36] Hansel T T, Kropshofer H, Singer T, Mitchell J A and George A J T, 2010, The safety and side effects of monoclonal antibodies, *Nat. Rev. Drug Discov.*, **9**: 325–38.
- [37] Van der Sloot A M, Tur V, Szegezdi E, Mullally M M, Cool R H, Samali A, Serrano L and Quax W J, 2006, Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor., *Proc. Natl. Acad. Sci. USA*, **103**: 8634–9.
- [38] Van der Sloot A M and Quax W J, 2011, Computational design of TNF ligand-based protein therapeutics, *Proc. Natl. Acad. Sci. USA*, **108**: 521–34.
- [39] Reis C R, Van Der Sloot A M, Szegezdi E, Natoni A, Tur V, Cool R H, Samali A, Serrano L and Quax W J, 2009, Enhancement of antitumor properties of rhTRAIL by affinity increase toward its death receptors, *Biochemistry*, **48**.
- [40] Reis C R, Van Der Sloot A M, Natoni A, Szegezdi E, Setroikromo R, Meijer M, Sjollem K, Stricher F, Cool R H, Samali A, Serrano L and Quax W J, 2010, Rapid and efficient cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL variants, *Cell Death Dis.*, **1**.
- [41] Shibata H, Yoshioka Y, Ohkawa A, Minowa K, Mukai Y, Abe Y, Tanai M, Nomura T, Kayamuro H, Nabeshi H, et al., 2008, Creation and X-ray structure analysis of the tumor necrosis factor receptor-1-selective mutant of a tumor necrosis factor- $\alpha$  antagonist, *J. Biol. Chem.*, **283**: 998–1007.
- [42] Wang Y, van Assen A H G, Reis C R, Setroikromo R, van Merkerk R, Boersma Y L, Cool R H, and Quax W J, 2017, Novel RANKL DE-loop mutants antagonize RANK-mediated osteoclastogenesis, *FEBS J.*, **284**: 2501–12.
- [43] Douni E, Rintoul V, Makrinou E, Zwerina J, Penninger J M, Eliopoulos E, Schett G and Kollias G, 2012, A RANKL G278R mutation causing osteopetrosis identifies a functional amino acid essential for trimer assembly in RANKL and TNF, *Hum. Mol. Genet.*, **21**: 784–798.
- [44] Reis C R, van Assen A H G, Quax W J and Cool R H, 2011, Unraveling the binding mechanism of trivalent tumor necrosis factor ligands and their receptors, *Mol. Cell. Proteomics*, **10**:

M110.002808.

- [45] Warren J T, Nelson C A, Decker C E, Zou W, Fremont D H, and Teitelbaum S L, 2014, Manipulation of receptor oligomerization as a strategy to inhibit signaling by TNF superfamily members, *Sci. Signal.*, **7**: ra80.
- [46] Arthur K K, Gabrielson J P, Hawkins N, Anafi D, Wypych J, Nagi A, Sullivan J K, and Bondarenko P V, 2012, In vitro stoichiometry of complexes between the soluble RANK ligand and the monoclonal antibody denosumab, *Biochemistry*, **51**: 795–806.